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Vacuolar H^+-Translocating ATPases from Plants: Structure, Function, and Isoforms

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The vacuolar H^+-translocating ATPase (V-type ATPase) plays a central role in the growth and development of plant cells. In a mature cell, the vacuole is the largest intracellular compartment, occupying about 90% of the cell volume. The proton electrochemical gradient (acid inside) formed by the vacuolar ATPase provides the primary driving force for the transport of numerous ions and metabolites against their electrochemical gradients. The uptake and release of solutes across the vacuolar membrane is fundamental to many cellular processes, such as osmoregulation, signal transduction, and metabolic regulation. Vacuolar ATPases may also reside on endomembranes, such as Golgi and coated vesicles, and thus may participate in intracellular membrane traffic, sorting, and secretion.

Plant vacuolar ATPases are large complexes (400–650 kDa) composed of 7–10 different subunits. The peripheral sector of 5–6 subunits includes the nucleotide-binding catalytic and regulatory subunits of ~70 and ~60 kDa, respectively. Six copies of the 16-kDa proteolipid together with 1–3 other subunits make up the integral sector that forms the H^+ conducting pathway. Isoforms of plant vacuolar ATPases are suggested by the variations in subunit composition observed among and within plant species, and by the presence of a small multigene family encoding the 16-kDa and 70-kDa subunits. Multiple genes may encode isoforms with specific properties required to serve the diverse functions of vacuoles and endomembrane compartments.

KEY WORDS: Vacuolar; H^+-ATPase; plant; transport; proton pump; tonoplast; V-type.

INTRODUCTION

In plant cells, electrogenic H^+ pumps play a central role in energizing the plasma membrane as well as the vacuolar membrane (Sze, 1985). A plasma membrane H^+-ATPase (P-type) extrudes H^+ from the cell, forming a membrane potential (negative inside) and a pH gradient (acid outside). Two distinct electrogenic H^+ pumps acidify the vacuolar compartment: a H^+-ATPase and a H^+-PPase (Rea and Sanders, 1987). The electrochemical gradient generated by these H^+ pumps provides the driving force for secondary transport of numerous ions and metabolites (Fig. 1). The membrane potential difference drives electrogenic uniport and the electrochemical gradient of H^+ drives transport of solutes in antiport or symport with H^+. In the last 6–7 years, remarkable progress has been made in understanding the structure and function of the vacuolar-type (V-type) H^+-ATPases from plants as well as animals (Forgac,

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[2]Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; CAM, Crassulacean acid metabolism; Nbd-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; Bz-ATP, 3-*O*-(4-benzoyl)benzolyadenosine 5'-triphosphate; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; NEM, *N*-ethylmaleimide; IP_3, inositol-1,4,5-triphosphate; H^+-PPase, H^+-translocating pyrophosphatase; V-type, vacuolar-type; P-type, phosphorylated intermediate- or plasma membrane-type; F-type, F_1F_o-type; V-ATPase, vacuolar-type H^+-ATPase.

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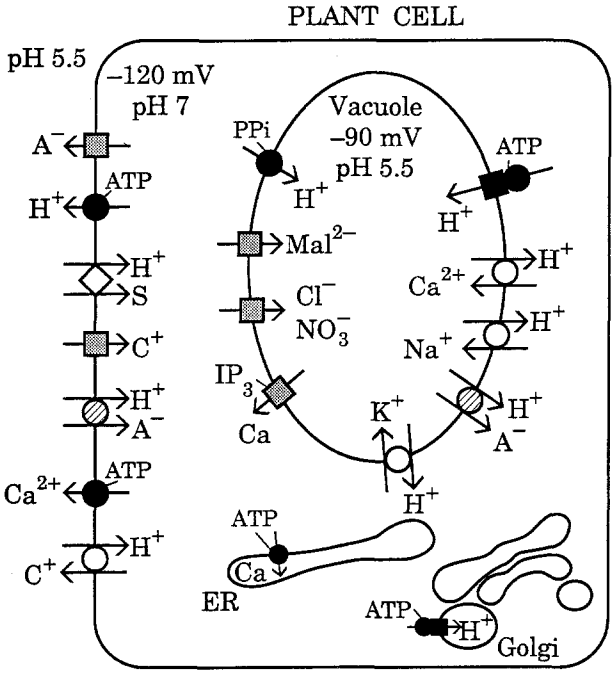

Fig. 1. Model of primary H^+ pumps, H^+-coupled transporters, and channels in a simplified plant cell. A plasma membrane ATPase (P-type) pumps H^+ out of the cell, generating a proton electrochemical gradient (inside −120 mV relative to the outside). An electrogenic H^+-ATPase (V-type) and a H^+-PPase acidify the vacuole. The protonmotive force provides energy for uptake and release of solutes across the tonoplast through antiporters (○), symporters (⊘), and channels (□). Ion pumps denoted by ●. C^+, A^-, and S refer to cations, anions, and organic solutes, respectively.

1989). This minireview highlights the biochemical and molecular properties of the plant V-ATPase and illustrates the vital role of this H^+ pump in the growth and development of plants.

A H^+ ELECTROCHEMICAL GRADIENT IS CENTRAL TO THE FUNCTIONS OF THE VACUOLE

Although meristematic plant cells contain numerous small vacuoles, in mature cells the vacuole is the largest intracellular compartment, occupying about 90% of the cell volume. Plant vacuoles are dynamic organelles which participate in a variety of cellular processes depending on the stage of development and the external signals received. These include transport and storage of ions and metabolites, osmoregulation, signal transduction, protein turnover, and regulation of cytoplasmic pH and $[Ca^{2+}]$. The protonmotive force generated by either the H^+-translocating inorganic pyrophosphatase (H^+-PPase) or the vacuolar-type H^+-ATPase (Rea and Sanders, 1987) can be used to drive secondary solute transport. The resulting ion and metabolite fluxes across the vacuolar membrane (or tonoplast) are central to the diverse functions of the vacuole in plant cells (MacRobbie, 1979).

Regulation of Cell Turgor

One of the most important functions of vacuoles is to generate and maintain turgor pressure, the driving force for cell expansion. With rigid cell walls, plant cells generate turgor pressure by maintaining a higher osmotic pressure than that of the intercellular space or the external medium. Turgor pressure increases as various osmotic solutes accumulate in the cell, which is followed by water uptake. K^+, Cl^-, and organic acids such as malate normally comprise the major osmotically active solutes. Although the mechanism involved is not clear, the control of turgor pressure must therefore involve the activation of H^+ pumps, H^+-coupled transporters, and ion channels at the plasma membrane and the tonoplast.

Changes in turgor pressure of guard cells regulate the opening and closing of stomatal pores in leaf epidermis which control gas exchange for photosynthesis (Schroeder and Hedrich, 1989). Guard cells of closed stomata contain many small vacuoles, while those of open stomata contain a large central vacuole typical of mature cells (MacRobbie, 1979). Accumulation of K^+ and malate in the vacuoles results in osmotic swelling and stomatal opening (Raschke *et al.*, 1988). Malate accumulation in vacuoles is probably driven by the membrane potential (positive inside), while K^+ could be accumulated via a H^+/K^+ antiport (Sze, 1985). Blue light-induced stomatal opening is caused in part by the activation of the plasma membrane H^+-ATPase (Assmann *et al.*, 1985). Hyperpolarization of the cell drives K^+ influx from the intercellular space into the cytoplasm. It is not known if the vacuolar H^+-ATPase is similarly activated to provide an increase in the driving force for ion uptake at the tonoplast.

Plants adapt to osmotic stress by adjusting their transport activities at the tonoplast. Sugar beet cells, grown in 200 mM NaCl, displayed a rapid induction of Na^+/H^+ antiport activity (Blumwald and Poole, 1987). Na^+ accumulation into the vacuole was dependent on the protonmotive force generated by the H^+-ATPase. In NaCl-adapted tobacco cells, both

ATP hydrolysis and H^+-transport activities increased 4-fold per unit of 69-kDa subunit (Reuveni *et al.*, 1990). In addition, the messenger RNA encoding the 69-kDa subunit increased 2-4-fold by short-term (24 h) treatment with NaCl (Narasimhan *et al.*, 1991). The relative activity of the vacuolar H^+-ATPase as seen by lead phosphate deposits was enhanced 3- 4-fold in vacuoles of tomato seedling roots grown in 150 mM NaCl (Sanchez-Aguayo *et al.*, 1991). These results indicate that induction of both the vacuolar H^+-ATPase and Na^+/H^+ antiport activities is involved in osmotic adjustment in plant cells.

Transport and Storage of Ions and Metabolites

Apart from osmoregulation, vacuoles participate in metabolic regulation through the storage and release of inorganic and organic solutes. Anions, such as NO_3^- and Cl^-, are found at higher concentrations in vacuoles than in the cytoplasm (Luttge and Higinbotham, 1979). The accumulation of Cl^- and NO_3^- into vacuoles is driven by the electrical potential (+ inside relative to the cytoplasm). Using tonoplast vesicles, anion influx could be measured indirectly as the dissipation of the membrane potential formed by electrogenic H^+ pumping (Kaestner and Sze, 1987) or by a Cl^--sensitive fluorescent probe (Pope and Leigh, 1990). Voltage-dependent ion channels, as detected by the patch-clamping of whole vacuoles, may mediate this anion uptake. These channels opened at positive membrane potentials (Hedrich and Neher, 1987); however, they transported both anions and cations. Although the resting membrane potential across the tonoplast is usually $+30 \pm 10$ mV (positive inside), transient voltage changes might occur in response to signals which activate H^+ pumps, channels, or both.

Vacuolar pH *in vivo* may be regulated in part by anion channels, since electrophoretic uptake of anions dissipates the electrical potential across the tonoplast and increases the pH gradient (Sze, 1985). The coordination of H^+ pump activity and anion channels would thus regulate the driving force (electrical potential or pH gradient) required for secondary transport.

In Crassulacean-acid-metabolism (CAM) plants, malate is taken up, stored, and released from vacuoles, as part of a mechanism to minimize water loss to the atmosphere. CAM plants, such as *Kalanchoe*, fix CO_2 as malic acid at night (Luttge, 1987). Initially stored in vacuoles, malic acid is then released and used in the day for photosynthesis. $Malate^{2-}$ accumulation, probably via a channel, is driven by the transmembrane electrical potential generated by the vacuolar H^+-ATPase (or H^+-PPase) (White and Smith, 1989). The mechanism of regulation of this diurnal flux of malate is not known at this time but may involve regulation of either the vacuolar H^+-ATPase, the malate channel, or both. In *Mesembryanthemum crystallinum*, CAM can be induced by drought or salinity. During CAM induction, vacuolar ATPase activity increases 4-fold. Interestingly, this was accompanied by alterations in the subunit composition of the vacuolar H^+-ATPase (Bremberger *et al.*, 1988).

The vacuoles of leaf mesophyll cells store sucrose and amino acids when synthesis exceeds export from the leaves (Boller and Wiemken, 1986). At least three amino acid transport systems exist at the tonoplast of barley mesophyll cells. Aromatic L-amino acids (Phe, Trp, Tyr) and leucine are accumulated in isolated vacuoles via H^+-coupled transport driven by either the H^+-ATPase or H^+-PPase (Homeyer *et al.*, 1989). However, two other amino acid transporters, with broad specificity, catalyze exchange of amino acids between the vacuole and cytoplasm (Martinoia *et al.*, 1991). In this case, ATP stimulates uptake as an effector but not as an energy source.

In storage tissues of sugar beets and sugarcane stalks, sucrose is stored in the vacuole. Some evidence suggests sucrose uptake into isolated vacuoles or tonoplast vesicles depends in part on a H^+/sucrose antiport driven by the vacuolar H^+-ATPase (Getz, 1991; Briskin *et al.*, 1985); however, most of the sucrose is taken up passively.

Regulation of Cytoplasmic $[Ca^{2+}]$ and pH

Many stimulus-induced responses in plant cells are coupled to transient changes in cytoplasmic $[Ca^{2+}]$ which is usually maintained at about 0.3 μM. The vacuole is a major reservoir of Ca^{2+}, and intravacuolar $[Ca^{2+}]$ can reach 0.1–10 mM (Evans *et al.*, 1991; Johannes *et al.*, 1991). Ca^{2+} is accumulated into vacuoles or tonoplast vesicles via a Ca^{2+}/H^+ antiporter driven by a pH gradient (acid inside) formed by the vacuolar H^+-ATPase (Schumaker and Sze, 1986; Guern *et al.*, 1989; Evans *et al.*, 1991). When the pH gradient was fixed, a positive membrane potential stimulated Ca^{2+} uptake, indicating a stoichiometry of $> 3H^+ : 1Ca^{2+}$ for the antiporter (Blackford *et al.*, 1990). Hence, it is thermodynamically possible that

the antiporter could achieve accumulation ratios of 10^5: 1 observed *in vivo*.

Calcium stored in the vacuole can be released via channels in response to signals, such as inositol 1,4,5-trisphosphate (IP_3) or voltage changes. Although the stimuli for IP_3 formation in plants is not known, IP_3-induced Ca^{2+} release has been detected in isolated tonoplast vesicles (Schumaker and Sze, 1987; Johannes *et al.*, 1991) or by patch-clamp of whole vacuoles (Alexandre *et al.*, 1990). This Ca^{2+} channel is activated by voltage (+10 to +80 mV relative to the cytosol). Another voltage-activated Ca^{2+} channel has recently been reported on the tonoplast (Johannes *et al.*, 1991). The ability to accumulate and release Ca^{2+} provides compelling evidence that vacuoles could play a major role in regulating cytoplasmic [Ca^{2+}].

Tonoplast transport activities also contribute to the maintenance of cytoplasmic conditions, including pH homeostasis (Guern *et al.*, 1991) and ionic conditions suitable for enzymes (MacRobbie, 1979). The role of the vacuolar H^+-ATPase in regulating cytoplasmic pH and [Ca^{2+}] was recently demonstrated in yeast. Mutants in which genes encoding V-ATPase subunits were disrupted could no longer grow in medium containing high or low [Ca^{2+}] at pH 5.5, while wild type cells grew at normal rates (Noumi *et al.*, 1991). The primary H^+ pumps create a driving force and the secondary transport of major anions or cations across the tonoplast ultimately regulates cytoplasmic pH (Guern *et al.*, 1991).

Storage of Proteins

Many types of proteins are stored in plant vacuoles. Storage proteins in seeds (e.g., corn or soybean) provide nutrition to the seedlings during germination. To protect against herbivores, some plants accumulate defense proteins that are inhibitors of animal enzymes (e.g., legume lectins). Mechanical wounding by insects or pathogens induces accumulation of defense proteins, such as protease inhibitors in tomato leaves (Ryan, 1990) and enzymes that degrade fungal cell walls, like chitinase and beta 1,3-glucanase. According to one model, during pathogen attack, the vacuolar enzymes are released when the host cell lyses and thus prevent further invasion by the pathogen.

Plant vacuoles from many tissues contain a diverse array of acid hydrolases (Boller and Wiemken, 1986), including phosphatases, glycosidases, nucleases, lipases, and proteases. In addition to the role in defense discussed above, these enzymes are thought to serve several purposes depending on the stage of development: (i) to digest storage proteins following sorting to protein bodies during germination, (ii) to digest cytoplasmic contents in an autophagic process during vacuole ontogeny; (iii) to facilitate protein, carbohydrate, or lipid turnover during growth, and (iv) to degrade cytoplasmic components during senescence. The vacuolar H^+-ATPase maintains an acidic environment optimal for activity of these hydrolases, so they can effectively digest their substrates.

V-TYPE H^+-ATPases IN OTHER ENDOMEMBRANES: GOLGI AND COATED VESICLES

Vacuoles are an integral part of the endomembrane system which includes the endoplasmic reticulum, Golgi network, coated vesicles, secretory vesicles, plasma membrane, nuclear envelope, as well as a variety of transition vesicles (Harris, 1986). The endomembrane system plays a major role in the biogenesis of organelles, the deposition of materials within the organelles, and the biosynthesis and transport of material destined for extracellular secretion. For example, seed storage proteins or acid hydrolases are synthesized at the ER, and then transported and deposited in the vacuole. Plant cells secrete a variety of proteins and polysaccharides into the extracellular space. Actively dividing cells build new walls by secreting matrix polysaccharides (pectins and hemicelluloses) that accumulate in the Golgi prior to secretion. To facilitate penetration into the soil, root cap cells secrete slime polysaccharides which are synthesized initially in the ER, and modified in the Golgi before exocytosis. In germinating barley, starch in the endosperm is hydrolyzed by alpha-amylase which is secreted from the aleurone layer.

Evidence that V-ATPases are an integral part of the endomembrane system in plants is emerging. Based on comigration of marker enzymes on sucrose gradients, V-type H^+-ATPases are associated with Golgi membranes of corn coleoptile and sycamore cells (Chanson and Taiz, 1985; Ali and Akazawa, 1986). These putative Golgi-associated ATPases are less sensitive to inhibition by NO_3^- than the tonoplast H^+-ATPase. Immunogold labeling with antibodies against the 70-kDa subunit decorated both the tonoplast and Golgi membranes of root tip cells from corn (Hurley and Taiz, 1989).

Table I. Properties of the vacuolar H^+-pumping ATPase from Oat Roots and Other Plant Tissues

Parameter	H^+-Translocating ATPases		
	Vacuolar	Mitochondrial	Reference[b]
H^+ : ATP stoichiometry	$2H^+$/ATP	$3H^+$/ATP	1, 2
Anion stimulation	Cl^- (K_m = 1 mM)	HCO_3^-	3–5
Substrate specificity	ATP > GTP	ATP > GTP	3, 4
K_m ATP	0.1–0.2 mM	0.8 mM	3, 4
pH optima	7.0 and 8.0	8.0–9.0	3, 4
Inhibitor sensitivity[a]			
Vanadate	No	No	3, 5
Azide	No	Yes (< 50 μM)	3, 5
NO_3^-	Yes (2–10 mM)	Yes (7 mM)	3, 4
Bafilomycin	Yes (2–3 nM)	No	5, 6
DCCD	Yes (3–4 μM)	Yes (0.04 μM)	3, 4
Nbd-Cl	Yes (2 μM)	Yes (50 μM)	3, 4
DIDS	Yes (5 μM)	Yes (5 μM)	3, 4

[a]Concentrations required for 50% inhibition.
[b](1) Bennett and Spanswick (1984); (2) Guern *et al.* (1989); (3) Wang and Sze (1985); (4) Randall and Sze (1986); (5) Ward and Sze, 1992a, b; (6) Bowman *et al.* (1988a).

Clathrin-coated vesicles in plants could participate in protein transport between the Golgi and storage vacuoles. A lectin precursor in developing pea cotyledons is associated with clathrin-coated vesicles (Harley and Beevers, 1989). Although clathrin-coated vesicles in animal cells are acidified by a V-type H^+-ATPase (Forgac, 1989), ATP-dependent H^+ transport in such vesicles from plants has not been demonstrated. However, a recent study showed staining of coated vesicles from zucchini hypocotyl with antibodies against the 70-kDa subunit of corn H^+-ATPase (Depta *et al.*, 1991). These results indicate that vacuolar-type H^+-ATPases are present on organelles of the secretory system, and that acidification of these compartments may be essential for protein sorting in plants, as has been observed in animal cells (Forgac, 1989).

PLANT VACUOLAR H^+-ATPases

Characteristics of Plant V-ATPase *in vitro*

The inhibitor sensitivity of the plant vacuolar ATPase clearly identified it as a novel H^+ pump, similar yet distinct from the F_1F_o-ATPase (Sze, 1985; Rea and Sanders, 1987; Table I). ATP hydrolysis and H^+ pumping activities of the vacuolar ATPase were initially examined using tonoplast vesicles or whole vacuoles (Sze, 1985) and more recently by patch clamp studies of whole vacuoles (Hedrich and Schroeder, 1989). The V-ATPase is insensitive to inhibitors of the P-type H^+-ATPase (vanadate) or the F-type mitochondrial H^+-ATPase (azide, oligomycin). The diagnostic inhibitors of the vacuolar H^+-ATPase are NO_3^- (in the presence of azide) and bafilomycin, a potent and specific inhibitor belonging to a class of macrolide antibiotics (E. J. Bowman *et al.*, 1988a). Bafilomycin A_1 at 2–3 nM inhibits 50% of both H^+-transport and ATP hydrolysis activity of the oat vacuolar H^+-ATPase. We estimated that one molecule of bafilomycin is sufficient to completely inhibit one molecule of ATPase (Ward and Sze, 1992a); however, the mode and site of action are not known.

Anions regulate the activity of the vacuolar H^+-ATPase in several ways: (i) stimulation by Cl^-, and (ii) inhibition by NO_3^-. At low concentrations (1–10 mM), NO_3^- inhibition of the red beet V-type H^+-ATPase was pseudo-competitive and reversible, while inhibition at higher concentrations of NO_3^- (250–500 mM) was attributed to dissociation of the enzyme complex (Rea *et al.*, 1987a). In patch clamp studies of whole vacuoles, NO_3^- applied to the lumenal side had no effect. However, NO_3^- inhibition of sugar beet vacuolar H^+-ATPase was partially reversible, suggesting NO_3^- acts on the cytoplasmic side of the membrane (Hedrich *et al.*, 1989). Cytoplasmic [NO_3^-] can reach 3–5 mM (Zhen *et al.*, 1991); therefore, [NO_3^-] may regulate H^+-pumping ATPase *in vivo*.

Chloride (K_m = 1 mM) directly stimulates the

vacuolar H^+-ATPase from plants, independently of its ability to dissipate the positive membrane potential and increase pH gradient formation (Sze, 1985). Chloride stimulated ATP hydrolysis of native vesicles in the absence of a membrane potential (Churchill and Sze, 1984) and of the detergent-solubilized enzyme (Bennett and Spanswick, 1983). Recently, we showed that Cl^--stimulated ATP hydrolysis of the purified enzyme (Randall and Sze, 1986) was coupled to H^+ transport in the purified and reconstituted H^+-ATPase when the electrical potential was clamped to zero (Ward and Sze, 1992b). These results clearly demonstrate that the H^+-ATPase from plants is stimulated directly by Cl^-. As the cytoplasmic $[Cl^-]$ is about 30–90 mM (Luttge and Higinbotham, 1979), stimulation of the V-ATPase activity *in vivo* would be optimal. Partial protection by Cl^- of the purified oat H^+-ATPase from NO_3^- inhibition (Randall and Sze, 1986) would suggest that Cl^- and NO_3^- may interact at the same anion-binding sites to modulate H^+ pumping into vacuoles *in vivo*.

The H^+/ATP stoichiometry of the plant vacuolar H^+-ATPase determined using either whole vacuoles (Guern *et al.*, 1989) or tonoplast vesicles (Bennett and Spanswick, 1984) is $2H^+$/ATP. Based on thermodynamic considerations, the H^+-ATPase should function in a hydrolytic mode even in the presence of a pH gradient of 3.5 (Rea and Sanders, 1987). Consistent with this prediction, the H^+-ATPase of sugar beets was capable of pumping against a pH gradient of 4 (acid inside) as determined in patch clamp studies using whole-vacuole configuration (Hedrich *et al.*, 1989).

Plant V-ATPase is a Large Complex with 7–10 Subunits

The subunit composition of vacuolar H^+-ATPases purified from several plants is similar in complexity to those found in animal and fungal endomembranes (Table II). The three major polypeptides (67–70, 55–60, and 16–17 kDa) initially identified as subunits are common to all V-type ATPases (Mandala and Taiz, 1985; Randall and Sze, 1986; Manolson *et al.*, 1985; Forgac, 1989). While the subunit compositions of the H^+-ATPase from red beet, mung bean, barley, and oat are similar in general, several important differences exist. The red beet and barley H^+-ATPases contain a 100–115-kDa subunit (Parry *et al.*, 1989; DuPont and Morrisey, 1992), which appears to be lacking in the H^+-ATPases from oat (Ward and Sze, 1992a) and mung bean (Matsuura-Endo *et al.*, 1990). This is of particular interest since the 100-kDa subunit of the bovine coated vesicle H^+-ATPase is required for H^+ transport activity (Adachi *et al.*, 1990). A 100-kDa polypeptide is clearly not required for the pump from oats, since the purified and reconstituted H^+-ATPase catalyzed electrogenic H^+ transport with its native properties (Ward and Sze, 1992b). Perhaps this subunit is species- or tissue-specific, or developmentally regulated. Other differences in subunit composition among plant V-type H^+-ATPases include the presence of a 52-kDa subunit in the red beet H^+-ATPase, but not a 34–37-kDa subunit found in other species (Table II). The variations in subunit compositions (such as the 100–115 kDa) would suggest the presence of several subtypes of V-ATPases in plants.

Assuming the stoichiometry of the plant V-ATPase is similar to that from brain coated vesicles (Forgac, 1989, see Table II), there would be three copies each of the 70- and 60-kDa subunit, six copies of the 16-kDa (Kaestner *et al.*, 1988), and one copy each of the remaining subunits. This stoichiometry would be consistent with a molecular mass of 590 kDa (*Kalanchoe*) to 690 kDa (oat, beet), which is close to 400–650 kDa estimated by radiation inactivation or gel filtration (Table II).

V-ATPase Complex Consists of a Peripheral and an Integral Sector

The multimeric enzyme is composed of a large peripheral sector and a membrane integral sector (Fig. 2). The peripheral sector of the enzyme complex is dissociated from the membrane with chaotropic reagents (e.g., KI) which inactivate ATP hydrolysis and H^+ transport activities. Six polypeptides of 70, 60, 44, 42, 36, and 29 kDa from oat (Ward *et al.*, 1992a; Lai *et al.*, 1988), and five polypeptides of 67, 55, 52, 44, and 32 kDa from red beet (Rea *et al.*, 1987a; Parry *et al.*, 1989) were solubilized and thus identified as peripheral. Five polypeptides from the oat ATPase were released by KI as a complex of 540 kDa. Surprisingly, the 42-kDa subunit is not associated with this complex but is released separately after dissociation (Ward and Sze, 1992a). Unlike the F_1-ATPase, the solubilized peripheral sector is inactivated by dissociation and unable to hydrolyze ATP.

Like V-type ATPases from animal sources (Moriyama and Nelson, 1989; Arai *et al.*, 1989), chaotrope-induced dissociation of plant vacuolar

Table II. Subunit Composition and Function of Vacuolar H^+-ATPases from Plant Tissues and Bovine Coated Vesicles

Source (M_r, kDa)	Subunit composition (kDa)												References[d]
Red beet	100	67	55[b]	52	44				32	16[c]			1
Mung bean		68	57		44	38	37	32		16	13	12	2
Barley	115	68	53		45	42	34	32		17	13	12	3
Oat (650)		70[a]	60		44	42	36	32	29	16[c]	13	12	4
Kalanchoe (510)		72	56	48		42			28	16			5
Corn (400)		72[a]	60							16[c]			6
Bovine coated vesicle (760)	100_1	73_3	58_3		40_1	38_1	34_1	33_1	19_1	17_6			7

[a] 70 kDa binds Nbd-Cl—catalytic subunit.
[b] 55 kDa binds Bz-ATP—regulatory subunit.
[c] 16 kDa binds DCCD—proteolipids form a channel for H^+ translocation.
[d] (1) Parry *et al.* (1989); Manolson *et al.* (1985); Rea *et al.* (1987a). (2) Matsuura-Endo *et al.* (1990). (3) DuPont and Morrissey (1992). (4) Randall and Sze (1987); Kaestner *et al.* (1988); Ward and Sze (1992a,b). (5) Bremberger *et al.* (1988); (6) Mandala and Taiz (1985, 1986). (7) Arai *et al.* (1988); Forgac (1989).

H^+-ATPases is enhanced by Mg^{2+} and ATP. Dissociation of the plant ATPase is temperature sensitive, occurring more rapidly at 4°C than at 20°C (Ward *et al.*, 1992; Parry *et al.*, 1989). MgATP interacts with a high-affinity binding site (K_d = 34 μM) of the H^+-ATPase from oats (Ward *et al.*, 1992). The nucleotide-binding site involved in enhancing dissociation may be at a catalytic site of the 70-kDa subunit or at the ATP-binding site of the 60-kDa subunit.

The peripheral sectors of the plant H^+-ATPase are visible on the surface of membrane vesicles as knob-like structures, 9–10 nm in diameter by electron microscopy (Fig. 2B). They are removed by KI or KNO_3, supporting their identity as the peripheral sector of the V-ATPase (Morre *et al.*, 1991; Klink and Luttge, 1991; Ward and Sze, 1992a). The surface view reveals a patchy distribution of structures containing six subparticles which may represent three copies each of the 70- and 60-kDa subunits (Taiz and Taiz, 1991; Morre *et al.*, 1991). The structure of the vacuolar H^+-ATPase is strikingly similar to that of F_1F_o-ATPases (Tiedge and Schafer, 1989). The knobs were observed at a higher density in membranes of *Mesenbryanthemum crystallinum* (Klink and Luttge, 1991) than in oats where the V-ATPase is estimated to comprise 30% and 8% of the membrane proteins, respectively. The other peripheral subunits (44, 42, 36, 29 kDa in oat) are proposed to form the stalk (Fig. 2A). However, the relative positions of these subunits have not been defined, and the subunit(s) which attach the peripheral to the membrane integral sectors is unknown.

The major component of the integral sector common to all V-ATPases is the DCCD-binding 16-kDa subunit, present in about six copies per holoenzyme (Kaestner *et al.*, 1988). This hydrophobic polypeptide is referred to as a proteolipid because of its solubility in chloroform/methanol (Rea *et al.*, 1987b). The 16-kDa subunit together with the 32-, 13-, and 12-kDa subunits of oat V-ATPase are considered part of the integral sector as they are not solubilized by chaotropic ions (Fig. 2A; Ward and Sze, 1992a). Interestingly, the integral complex of the red beet V-ATPase includes the 16-kDa subunits plus a 100-kDa polypeptide (Parry *et al.*, 1989).

The dissociation and inactivation of the oat H^+-ATPase by KI is reversible (Ward *et al.*, 1992). Removal of KI and MgATP by dialysis caused reassembly of the complex into a fully functional H^+ pump similar to the bovine coated vesicle H^+-ATPase (Puopolo and Forgac, 1990). In the oat V-ATPase, perhaps the 42-kDa peripheral subunit, which is solubilized and separated from the large peripheral sector during dissociation, functions as the attachment site of the two sectors. It is unclear whether such reversible dissociation of the H^+-ATPase in animal cells has any physiological implications; in plants, enzymes dissociate in response to chilling injury. Mung bean suspension culture cells incubated at 2°C rapidly lose both vacuolar ATPase and H^+ transport activities (Yoshida, 1991). Full activity is restored within 1 h once the cells are returned to 22°C. Recovery does not require *de novo* protein synthesis, suggesting reassembly of the enzyme complex.

Assignment of Subunit Functions

The major ~70-kDa subunit has a nucleotide-binding site which is probably the site of ATP hydrolysis (catalytic site). Nbd-Cl (an adenine analog)

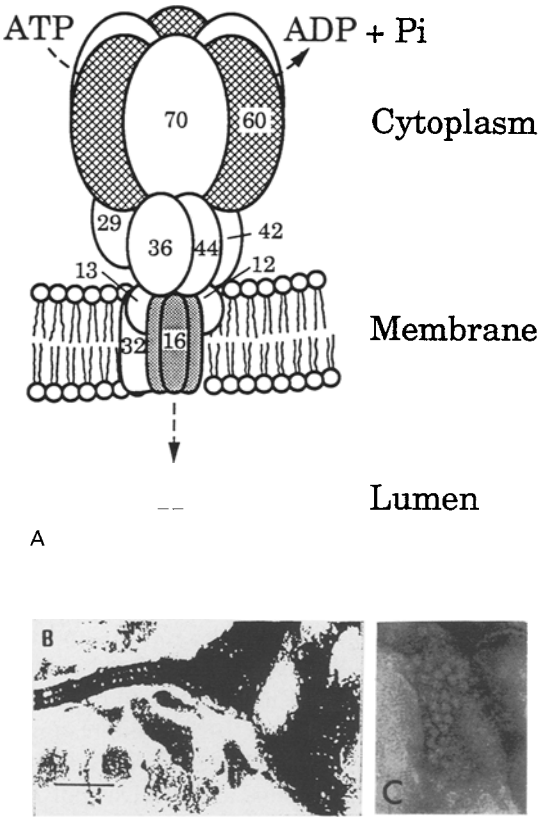

Fig. 2. Structure of a vacuolar-type H^+-ATPase from oat roots. (A) Structural model. A large peripheral complex includes the 70-kDa (catalytic) and the 60-kDa (nucleotide-binding) subunits plus several accessory subunits of yet unknown function. Six copies of the DCCD-binding 16-kDa proteolipid together with other integral subunits are thought to form a pore for H^+ transport. (B) V-ATPases appear as knob-like structures on membrane vesicles from oat roots. Side view: negatively stained vesicles reveal dense patches of knobs (~10 nm). (C) Surface view: knobs appear triangular, tetahedral, or hexagonal. Magnification: 190,000 ×. Bar = 100 nm.

or NEM inactivate the ATPase by binding to the 70-kDa subunit. Nbd-Cl binds near or at the catalytic site as its inhibition can be protected by the substrate, MgATP (Mandala and Taiz, 1986). A cysteinyl –SH or tyrosyl –OH was probably modified by this reagent as dithiothreitol reversed both Nbd-Cl binding and ATPase inactivation (Randall and Sze, 1987). The amino acid sequence of the 69-kDa subunit from carrot deduced from molecular cloning reveals regions with high homology among ~68-kDa subunits (Zimniak *et al.*, 1988; E. J. Bowman *et al.*, 1988b). Its role in catalysis is supported by the existence of regions homologous to the consensus sequences for nucleotide binding of several proteins, including the beta subunit of F_1-ATPases: G-X-X-X-X-G-K-T/S and G-X-T-X-A-E-X-X-D-X-G (Table III) (Walker *et al.*, 1982). However, it is not clear whether the Cys(C) and Tyr(Y) within these regions of the V-ATPase ~68 kDa (see Table III) are modified by Nbd-Cl or NEM.

The ~60-kDa subunit also has a nucleotide-binding site which is considered a regulatory site. The photoactivated ATP analogue, 3-*O*-(4-benzyoyl)benzoyladenosine 5′-triphosphate (Bz-ATP), binds to the 60-kDa subunit. Although it inactivates the ATPase by 50% at 10 μM, Bz-ATP is not a simple competitive inhibitor (Manolson *et al.*, 1985). The ability to bind ATP is supported by the presence of a putative nucleotide-binding sequence, A/G-X-X-X-G-M/K-T, present in the ~55–57-kDa subunit from both *Arabidopsis* and *Neurospora* (Manolson *et al.*, 1988; Bowman *et al.*, 1988a). A similar site exists in the alpha subunit of F_1-ATPases. Although this sequence is conserved and similar to "GXXXXGKT/S" found in the 67–69-kDa subunit, the other consensus sequence of "GXTXAEXXRDXG" is poorly conserved (Table III) (Inatomi *et al.*, 1989). The 70- and 60-kDa subunits are not only highly homologous with each other but also with the beta and alpha subunits, respectively, of the F_1F_o-ATP synthetase (Gogarten *et al.*, 1989). Taken together, the presence of nucleotide-binding sites in both 70- and 60-kDa polypeptides suggest they may participate in catalysis analogous to the alpha and beta subunits of the F_1F_o-ATPase (Forgac, 1989).

DCCD inhibition of ATPase activity is caused by its covalent modification of the 16-kDa proteolipid (Kaestner *et al.*, 1988; Rea *et al.*, 1987b). Although purification studies reveal multiple (about six) copies of the 16-kDa subunit per holoenzyme, activity is completely inhibited when only 1 mole of DCCD is bound per mole of ATPase (Rea *et al.*, 1987b; Kaestner *et al.*, 1988). The predicted amino acid sequence of the oat proteolipid revealed a molecule with four membrane-spanning domains (Lai *et al.*, 1991). Domain IV is especially conserved, showing ~80% amino acid sequence identity between the oat and yeast proteolipid (Table III). This domain is thought to contain amino acid residues involved in H^+ conductance. A glutamate residue (E) within the hydrophobic region is the putative DCCD-binding residue. Replacing glutamate-137 by several amino acids, except aspartate, abolished activity in yeast (Noumi *et al.*, 1991). Based on similarities between the

Table III. Properties of Plant and Fungal V-ATPase Subunits Deduced from Their Primary Structure[a]

Source	Mr (kDa)	Amino acids	Location	Sequence	Starting residue
Nucleotide-binding proteins				~20 aa ~50 aa	
Consensus sequence				GXXXXGKT/S.^.GER.^.GXTXAEXXRDXG	
E. coli beta (F-ATPase)	50.2	460	peripheral	GGAGVGKT...^.GER.^.GLTMAEKFRDEG	149
Consensus sequence				A/GXXXGK/MT	
E. coli alpha (F-ATPase)	55.3	513	peripheral	GEVKGKT	315
~70 kDa (subunit A)					
Carrot cDNA	68.8	623	peripheral	GAFGCGKT..^..GER.^.GITIAEYFRDQG	252
Neurospora gDNA	67.1	607	peripheral	GAFGCGKT..^..GER.^.GITVAEYFRDQG	246
Yeast *g*DNA	67	617	peripheral	GAFGCGKT..^..GER.^.GITLAEYFRDQG	258
~60 kDa (subunit B)					
*Arabidopsis c*DNA	55	492	peripheral	AIGEGMT	387
*Neurospora g*DNA	56.8	513	peripheral	AIGEGMT	380
~16 kDa					
Oat *c*DNA	16.6	165	membrane	QQPKLFVGMILILIFA*E*ALALYGLIVGIIL	126
Yeast *g*DNA	16.4	160	membrane	QQPRLFVGMILILIFA*E*VLGLYGLIVALLI	121

[a]Conserved sequences in the ~70- and ~60-kDa subunits of the V-ATPase are compared with consensus sequences for putative nucleotide-binding domains (Walker *et al.*, 1982; Inatomi *et al.*, 1989) also found in the alpha and beta subunits of *E. coli* F-ATPase. Conserved sequence in the 16-kDa proteolipid of V-ATPases is found in membrane-spanning domain IV which includes a glutamate residue (E). References: *Neurospora crassa* (B. J. Bowman *et al.*, 1988; E. J. Bowman *et al.*, 1988a); carrot (Zimniak *et al.* 1988); *Arabidopsis thaliana* (Manolson *et al.*, 1988); yeast 16 kDa (Nelson and Nelson, 1989); oat (Lai *et al.*, 1991); yeast 70 kDa (Hirata *et al.*, 1990); putative nucleotide-binding domains derived from several proteins (Walker *et al.*, 1982).

V-ATPase 16-kDa proteolipid and the 8-kDa proteolipid of the F_1F_o-ATPase, DCCD binding may block H^+ conductance by modifying the cooperative interaction required among the six proteolipids to form a functional proton pore. It is unclear whether other polypeptides in the integral sector are required to form the proton pore.

Unlike the F_o-ATPase (Forgac, 1989), the membrane integral sector of the V-ATPase does not passively conduct H^+ after removal of the peripheral sector. Tonoplast vesicles stripped of the peripheral components of the V-ATPase can hold a pH gradient generated by the vacuolar H^+-PPase (Rea *et al.*, 1987a; Ward and Sze, 1992a). This may be due to conformational changes in the integral sector after release of the peripheral sector, or a requirement for certain peripheral subunits to form the proton pore.

Aside from the 70-, 60-, and 16-kDa, the roles of the other subunits are not clear. Some may be required for attachment of the peripheral sector to the membrane, while others may be required for regulation, assembly, or sorting. For example, one form of the 31-kDa subunit from bovine kidney may have a role in sorting to the brush border (Wang and Gluck, 1990). The role of the subunits can be tested directly by gene disruption or by site-directed mutagenesis. These approaches have shown that the 70-, 60-, 31- and 16-kDa subunits are all essential for growth of yeast in medium at high pH (>7.5) or high [Ca] (Nelson and Nelson, 1990; Noumi *et al.*, 1991; Foury, 1990).

GENE FAMILIES AND ISOFORMS OF THE PLANT V-ATPase

Compelling evidence suggests that plants contain a family of V-type H^+-ATPases. A gene family of at least four members encodes the 16-kDa proteolipid subunit in oats (Lai *et al.*, 1991). The four distinct *c*DNA clones showed extensive divergence in their codon usage and their 3′-untranslated regions; however, the deduced amino acid sequences of the proteins were 97–99% identical. Southern analysis of the tobacco genome also demonstrated that multiple (four) DNA fragments hybridized with a *c*DNA encoding the 70-kDA subunit (Narasimhan *et al.*, 1991).

Biochemical evidence for distinct types of V-ATPases in plants exist in the plant literature. (i) Both Cl^--stimulated ATPase and H^+ pumping of

tonoplast vesicles from oat showed dual pH optima of 7 and 8 (Wang and Sze, 1985; Randall and Sze, 1986), suggesting the possibility of a mixture of V-ATPases. (ii) Two types of oat V-ATPases were separated after purification by ion exchange, judging by the variations in their polypeptide profiles (Ward and Sze, unpublished). (iii) Two-dimensional gel analysis revealed at least two spots that reacted with antibody to the 70-kDa subunit (DuPont *et al.*, 1988), and two ~60-kDa polypeptides were immunoprecipitated by antibody to the oat V-ATPase (Randall and Sze, 1989). (iv) Different types of V-ATPase may be associated with different organelles, like Golgi and coated vesicles (Chanson and Taiz, 1985; Depta *et al.*, 1991). (v) In a facultative CAM plant, the V-ATPase purified from plants in the C_3 state differed in subunit composition from that of the CAM state (Bremberger *et al.*, 1988).

SUMMARY

The protonmotive force generated by V-ATPases plays a major role in a number of cellular functions in plants. We have just begun to unravel the complex structure of V-ATPases in plants and to understand the roles of each subunit. Little is known about their physiological regulation. The presence of several types of V-ATPases in plants is intriguing. Perhaps the activity and biosynthesis of each type of V-ATPase is differentially regulated depending on its specific role and the stage of development. Multiple genes could encode isoforms with unique properties and levels of expression to serve the needs for an electrochemical proton gradient. While some genes of the V-ATPases are constitutively expressed (Struve *et al.*, 1990), others may respond to environmental or developmental stimuli (Thea Wilkins, personal communication). Gene-specific probes and promoter analyses will be useful tools to analyze the expression of tissue-specific or developmental-specific isoforms of H^+-ATPase subunits. Immunocytochemical staining using isoform-specific antibodies will help determine the distribution of different V-type H^+-ATPases in tissues, cells, and organelles. These studies will help us understand how vacuolar membrane energization and solute fluxes are integrated into cell functions.

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